Emixustat Reduces Metabolic Demand of Dark Activity in the Retina

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Purpose. In the dark, photoreceptor outer segments contain high levels of cyclic guanosine 3′-5′ monophosphate (cGMP), which binds to ion channels, holding them open and allowing an influx of cations. Ion pumping activity, which balances cation influx, uses considerable amounts of adenosine triphosphate (ATP) and oxygen. Light reduces cation influx and thereby lowers metabolic demand. Blood vessels are compromised in the diabetic retina and may not be able to meet the higher metabolic demand in darkness. Emixustat is a visual cycle modulator (VCM) that reduces chromophore levels and, therefore, may mimic light conditions. We evaluated the effect of emixustat on oxygen consumption and cation influx in dark conditions.

Methods. Cation influx was measured in rats using Mn^{2+}-magnetic resonance imaging (MEMRI). Retinal oxygen profiles were recorded to evaluate oxygen consumption. In the MEMRI protocol, animals were treated with either emixustat or vehicle. In the oxygen protocol, animals were untreated or treated with emixustat.

Results. In vehicle-treated animals, cation channel activity increased in the dark. Emixustat treatment reduced cation channel activity; activity was comparable to vehicle-treated controls in light conditions. In vehicle-treated animals, minimum retinal oxygen tension decreased as the retina recovered from a photobleach, indicating that more oxygen was being consumed. Emixustat treatment prevented the decrease in oxygen pressure after photobleach.

Conclusions. Emixustat reduced the cation influx and retinal oxygen consumption associated with dark conditions. VCMs are a promising potential treatment for ischemic retinal neovascularization, such as that in diabetic retinopathy.

Keywords: emixustat, retina, dark adaptation

Photoreceptors are unlike most neurons because in the unstimulated state/dark state, photoreceptors are depolarized and, therefore, release the neurotransmitter (glutamate). In the dark, photoreceptor outer segments contain high levels of cyclic guanosine 3′-5′ monophosphate (cGMP), which binds to ion channels, holding them open and allowing an influx of cations (mostly Na^+ and Ca^{2+}). To complete the current loop, there is an efflux of K^+ from the inner segment . This influx of Na^+ and Ca^{2+} ions and return efflux of K^+ is known as the dark current.[1] Large amounts of oxygen and adenosine triphosphate (ATP) are required to pump Na^+ back out and K^+ back in.[2] (Ca^{2+} is restored by an outer segment Na^+/Ca^{2+} exchanger, which adds to the Na^+ load on the cell[1].) Vessel damage associated with diabetic retinopathy (DR) reduces oxygen supply causing hypoxic conditions[3] that are exacerbated during dark adaptation.[4] Aberrant neovascularization, as in proliferative DR,[4] is probably a result of hypoxia.[5]

Phototransduction is initiated when a photon of light activates rhodopsin. Rhodopsin consists of opsin protein covalently bound to the chromophore, 11-cis-retinal. When light strikes 11-cis-retinal, it is converted to all-trans-retinal and opsin undergoes a series of conformational changes resulting in a form called metarhodopsin II (Meta II), which activates an associated G protein, transducin, which leads to the activation of cGMP phosphodiesterase (PDE6), which breaks cGMP down into 5′-GMP. Reduced cGMP allows the ion channels to close, preventing cation influx, hyperpolarizing the cell, and stopping the release of glutamate. All-*trans*-retinal is released from opsin creating apo-opsin. Apo-opsin can generate low level phototransduction (estimated activity 10^6-fold lower than that of Meta-II[6,7]).

Emixustat (CAS number 1141777-14-1, ACU-4429) reduces chromophore levels[8] and, therefore, may mimic a state of constitutive phototransduction through the accumulation of apo-opsin, thereby decreasing the dark current.[9,10] Previously, we demonstrated that emixustat reduced neovascularization in the oxygen induced retinopathy (OIR) model.[11] In this study, we investigated the hypothesis that emixustat puts the photoreceptors in a state of partial light adaptation and reduced metabolism by testing the accumulation of ions using Mn^{2+}-magnetic resonance imaging (MEMRI) and measuring retinal oxygen profiles in rats treated with emixustat.

Methods

Emixustat Hydrochloride

Emixustat hydrochloride (emixustat, chemical name: (*R*)-(+)-3-amino-1-(3-cyclohexylmethoxy)phenyl)propan-1-ol hydrochloride) is a nonretinoid, small molecule with a molecular weight of 299.84 g/mole ($C_{16}H_{25}NO_2$•HCl). Chemical synthesis of emixustat has been described by Bavik et al.[11]

Animals

Experiments using rats were approved by the institutional animal care and use committees of Vanderbilt University or Northwestern University, as appropriate, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

MEMRI Single Dose Study

Adult male Brown Norway Rats 3 months old (200–300 g) were treated by oral gavage with a single dose of 1 or 10 mg/kg emixustat, vehicle for emixustat (water), 200 mg/kg retinyl acetamide, or vehicle for retinyl acetamide (corn oil). Two hours after oral treatment, rats (five per group) were treated with topical tropicamide to dilate their pupils, photobleached (5000 Lux white light for 10 minutes), and treated with $MnCl_2$ (60 mg/kg, intraperitoneal [IP]). After 4 hours of dark adaption, MEMRI measurements were obtained following established protocols.[12,13] Light controls received the same treatments as the vehicle-treated rats, but remained in the light instead of undergoing 4 hours of dark adaptation.

MEMRI Multidose Study

Adult male Brown Norway Rats 3 months old (200–300 g) were dosed with 5 mg/kg/day emixustat or an equivalent volume of vehicle (water) by oral gavage. Rats were dosed twice-daily at lights on and at lights off for 6 days under conditions of normal cyclic light exposure (12 hours of approximately 100 lux of diffuse white fluorescent light). Immediately following the morning dose on day 7, rats received topical tropicamide to dilate pupils and were housed in ambient light for 6 hours. Following six hours in normal light, dark adapted rats received an intraperitoneal injection of $MnCl_2$ (60 mg/kg) and were dark adapted for 4 hours. The light-adapted rats received an intraperitoneal injection of $MnCl_2$ (60 mg/kg) and were left in ambient room light for 4 hours.

MEMRI Imaging

Rats were transported to the Vanderbilt University Institute of Imaging Science (VUIIS) using dark-adapted or light-adapted transport according to their respective experimental paradigm. During trial MRI measurements, we derived the following optimal protocol that was applied to all animals in each cohort. A snapshot FLASH inversion recovery imaging sequence was used to acquire a single imaging slice bisecting the retina in the axial and sagittal planes using a 12 mm inner diameter linear surface coil. The following imaging parameters were used: TR/TE = 1000/2.7 ms, inversion time = 125 ms, sweep width = 73.5 kHz, number of acquisitions = 32; slice thickness = 0.7 mm, field of view = 12 × 12 mm with 256 × 256 data matrix. These parameters resulted in an in-plane resolution of 47 μm. Image sequences were acquired at six inversion times to obtain a T1-weighed map (50, 150, 300, 400, 900, and 1800 ms). Animals in which movements were detected were reimaged after recovery. Sequential images were registered as necessary along the optic disc-corneal apex using MATLAB (Mathworks, Natick, MA, USA). Sequential images were registered as necessary along the optic disc-corneal apex using MATLAB.[14] T1 values were obtained from retinal MEMRI scans for the neural retina on a pixel-by-pixel basis from the T1 map. The T1 value for a retina was taken as the mean of all pixel values obtained for the retina, excluding 10% of values from the nasal and temporal edges. This was done to eliminate edge-effects. Due to limitation in resolution, we did not perform a layer-specific analysis, but rather averaged over tissue volume. This limitation likely underestimates the drug effect on the photoreceptors and outer retina. The area of interest was outlined manually by an observer blinded to the experimental conditions and MATLAB[14] protocols used to quantify pixel values as described. Choroid and vitreous signal was minimized by excluding the outer- and inner-most single pixel band of the area of interest, though we did not distinguish inner from outer retina. R1 is the inverse of T1 (1/R1)[14]; R1 values were for comparisons because R1 directly correlate with Mn^{2+} movement through channels.

Retinal PO_2 Measurements—Surgical Preparation of Rats

Methods were described previously by Lau and Linsenmeier.[15] Briefly, adult male Long Evans rats were anesthetized with isoflurane, and a tracheal tube, arterial cannula (for blood pressure and blood gas measurements), venous cannula for drug infusion, and Ag/AgCl reference electrodes in the neck were surgically implanted. Surgery also was done on the right eye so that it could be attached to a stabilizing plate, and penetrated with a needle to carry a microelectrode. During surgery, a loading dose of urethane was given slowly, and isoflurane was reduced to zero at least two hours before any recordings were made. Double barreled microelectrodes were made and calibrated by published methods.[16] One barrel is sensitive to oxygen and records a current proportional to local PO_2, and the other barrel records the local voltage, used here to measure vitreal and intraretinal electroretinograms (ERGs). An electrode was inserted through the needle in the eye and was sealed with a Silastic rubber "boot," which prevented leakage of vitreous but allowed the electrode to move back and forth under control of a hydraulic microdrive

Retinal PO_2 Measurements

Rats were treated with 1 mg/kg emixustat IV or not treated (n = 3/group) and after 30 to 60 minutes, rats were photobleached (1000 Lux white light for 15 minutes). Retinal oxygen profiles were recorded before bleaching and for 2.5 to approximately 3.75 hours after bleaching, all in the absence of light.

Results

Emixustat Reduced Retinal Dark Current

cGMP-gated cation channel activity in photoreceptors is regulated by phototransduction and more channels are open in the dark. Inhibition of RPE65 isomerase by emixustat reduces 11-*cis*-retinal levels, resulting in accumulation of apo-opsin. Accumulation of high levels of apo-opsin should bind enough transducin to activate the phototransduction cascade and PDE6, leading to cGMP hydrolysis, and closure of the ion channels responsible for the dark current. The reduction of visual cycle activity resulting from emixustat inhibition of RPE65 isomerase is expected to close the retinal cGMP-gated cation channels during dark adaptation. To test if emixustat

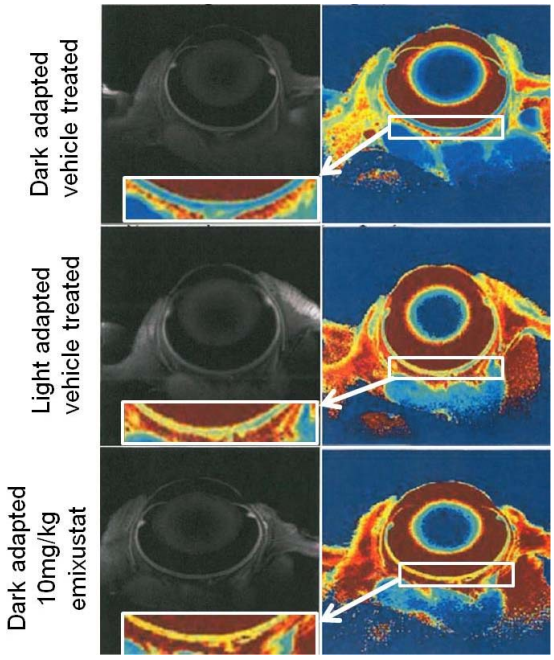

FIGURE 1. Pseudo-colored MEMRI images. Adult male Brown Norway Rats were treated with emixustat or vehicle (water) as indicated. Two hours later, rats were subjected to a photobleach and treated with the contrast agent $MnCl_2$. After 4 hours of dark adaption, MEMRI measurements were obtained. Light control animals (*middle row*) received the $MnCl_2$, but not emixustat treatment and remained in the light instead of undergoing 4 hours of dark adaptation. A change from *blue* to *yellow* represents a transition from high to low cation channel conductance. *Blue color* in the dark-adapted condition (*upper row*) is consistent with high channel conductance and the dark current. *Yellow color* in the light-adapted condition (*middle*) indicates reduced channel conductance. Treatment with emixustat mimics the light-adapted vehicle-treated condition.

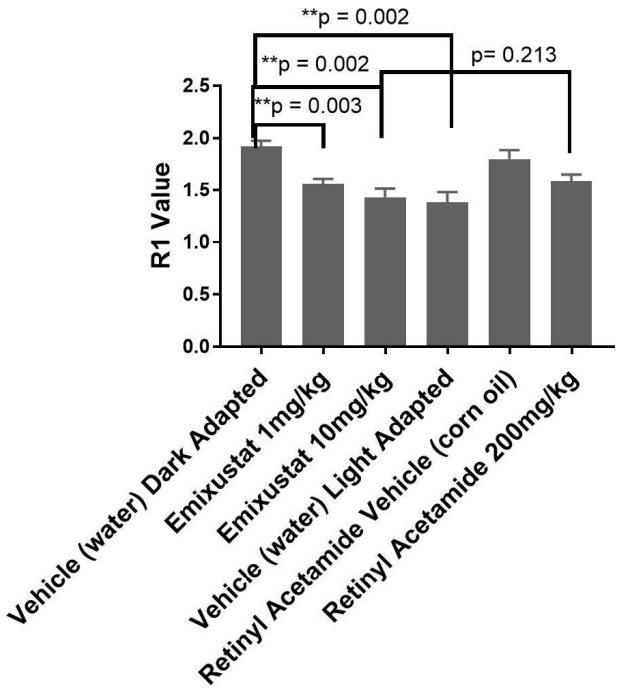

FIGURE 2. R1 values in vehicle-treated, dark-adapted animals were significantly higher than R1 values in vehicle-treated, light-adapted animals (1.92 vs. 1.38, respectively), consistent with high channel conductance during dark adaptation. Single doses of emixustat (1 and 10 mg/kg) produced statistically significant reductions in channel conductance (lower R1) during dark adaptation (1.56 and 1.43, respectively); these values were comparable to R1 values observed for vehicle-treated, light-adapted animals. Retinyl acetamide reduced channel conductance (R1 value = 1.59), but at a higher dose (200 mg/kg) compared to emixustat. Values shown are mean plus SD. The mean T1 value of each experimental group was measured for significance compared to other groups using standard *t*-tests; the distribution of T1 values was first checked for normality using the Shapiro-Wilk statistic. For all groups, $n = 5$.

could reduce the conductance of the retinal cation channels after dark adaptation, we performed Mn^{2+}-enhanced magnetic resonance imaging (MRI) analyses. Mn^{2+} is a potent cellular contrast agent used for MRI imaging and is also a Ca^{2+} analog and, therefore, surrogate marker for neuronal activity in the retina involving cation conductance including Ca^{2+}.[17]

Rats that were treated with a single dose of vehicle or emixustat were exposed to photobleach and then placed in the dark or ambient light. MEMRI images were obtained. In pseudo-color images from these animals, the blue color represents high cation concentration and yellow represents low cation concentration (Fig. 1). The blue color in the dark-adapted condition is consistent with high channel conductance and the high dark current. The light-adapted vehicle-treated retina has reduced activity and, therefore, is yellow. The emixustat-treated dark adapted retina mimics the light-adapted retina and, hence, is represented by yellow.

The pseudo-color images represent R1 values, which represent Mn^{2+} movement through ion channels and are proportional to the amount of cation channel conductance.[14] Low R1 values indicate less Mn^{2+} movement and, therefore, less channel conductance. A single dose of emixustat (1 and 10 mg/kg) produced statistically significant reductions in channel conductance (lower R1 values) during dark adaptation; these values were comparable to R1 values observed for vehicle-treated, light-adapted animals. Retinyl acetamide, which also is known to modulate visual cycle activity through inhibition of isomerase activity,[17] moderately reduced channel conductance at a higher dose (200 mg/kg), but not significantly different from emixustat nor its vehicle ($P \geq 0.21$; Fig. 2). These results suggested that emixustat may reduce the dark current. Since maintaining the dark current requires significant amounts of energy and oxygen, a treatment that results in reduced dark current could be beneficial for treatment of conditions in which retinal hypoxia and/or metabolic stress have roles.

In a similar, but multidose study without photobleaching, adult male Brown Norway rats were orally dosed twice per day with 5 mg/kg emixustat or an equivalent volume of vehicle (water) by oral gavage for 6.5 days. In this study, R1 values of the multidose emixustat-treated group were not significantly different from the vehicle dark-adapted group (Fig. 3). The difference in statistical significance between the two studies may be explained by high variability in the vehicle group of the multidose study. Another explanation may be due to the absence of photobleach in the multidose paradigm. The single dose study included a bleaching step that is expected to deplete the chromophore, regeneration of the chromophore then is inhibited by emixustat. This combination of bleach and emixustat creates and sustains high levels of apo-opsin. The multidose study did not include a bleaching step. Perhaps under these conditions, there was not sufficient signaling from apo-opsin to close a significant numbers of cation channels.

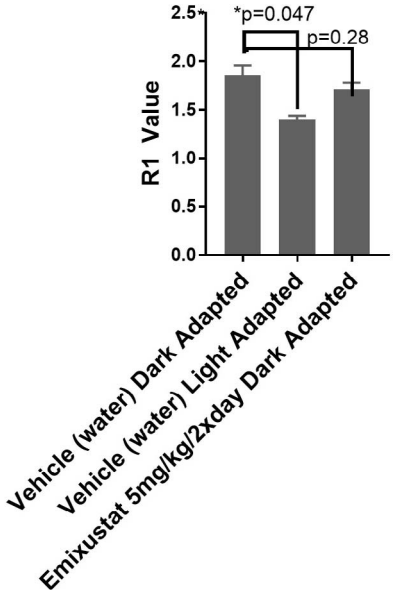

FIGURE 3. R1 values in vehicle-treated, dark-adapted animals were significantly higher than R1 values in vehicle-treated, light-adapted animals (1.86,1.40, respectively), consistent with high channel conductance during dark adaptation. Multiple doses of emixustat (5 mg/kg/day) produced less channel conductance (lower R1 values) during dark adaptation (1.71) than vehicle-treated animals; however, this change was not statistically significant. Values shown are mean plus SD. The mean R1 value of each experimental group was measured for significance compared to other groups using standard *t*-tests; the distribution of R1 values was first checked for normality using the Shapiro-Wilk statistic. For the emixustat treated group $n = 6$, for all other groups $n = 5$.

Emixustat Reduces Oxygen Consumption During Dark Adaptation

Photoreceptor oxygen use decreases during light adaptation and after photobleaching, leading to increased oxygen levels in the outer retina.[18,19] A decrease in outer retinal oxygen levels is expected as the retina returns to the dark-adapted state and uses more oxygen to support the metabolic activity associated with the dark current. As a result of increased oxygen consumption in the dark, PO_2 in the outer, avascular layers is lower in the dark.[2] Inhibition of RPE65 isomerase by emixustat is expected to decrease 11-cis-retinal levels, which would slow or prevent dark adaptation after photobleaching; this should keep the oxygen levels higher for a longer period than for untreated retinas. We examined the effect of emixustat on retinal oxygenation during recovery from photobleaching. Adult male Long Evans rats were treated with 1 mg/kg emixustat by intravenous injection. After 30 to 60 minutes, rats were exposed to bleaching light (1000 lux for 15 minutes). Oxygen levels were measured with microelectrodes in darkness before and after bleaching.

The profiles were recorded after inserting the microelectrode to the choroid. The indications that the electrode reached the choroid were a fluctuation in the voltage signal with the heart and respiratory rates, which occurs only when the electrode reaches the RPE and choroid, as well as a decrease in voltage when the electrode crosses the RPE (the transepithelial potential). The oxygen level was measured as partial pressure of oxygen (PO_2, in mm Hg) and recorded at multiple distances from the choroid as the electrode was withdrawn through the retina at 2 μm/sec (Fig. 4). The minimum PO_2 (Pmin), which corresponds to maximum oxygen consumption, occurred at the photoreceptor inner

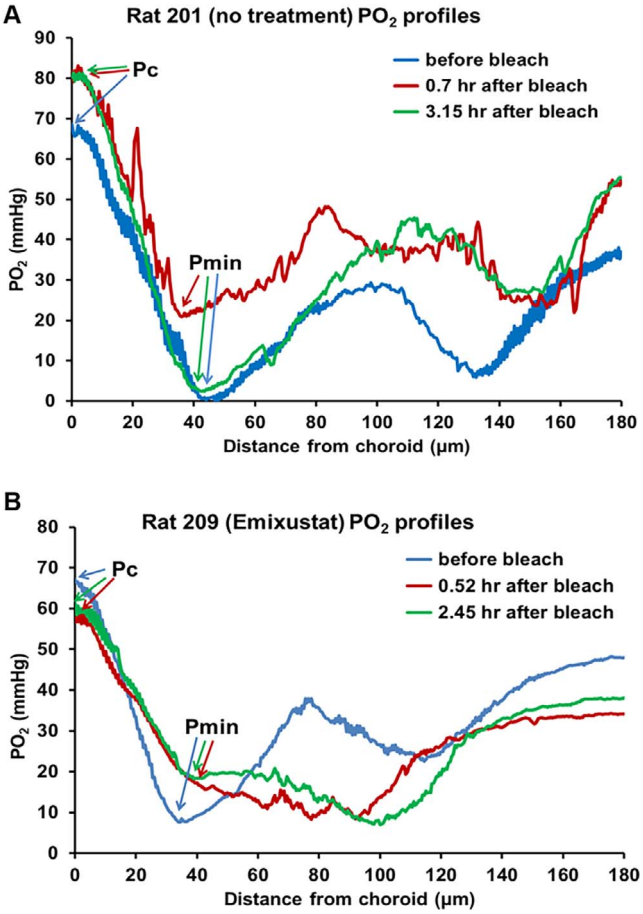

FIGURE 4. Representative PO_2 profiles. (**A**) From an untreated animal. (**B**) From an emixustat-treated animal. Rats were treated with 1 mg/kg emixustat (**B**) intravenously or not treated (**A**) and, after 30 to 60 minutes, were photobleached (1000 Lux white light for 15 minutes). Retinal oxygen profiles were recorded. *Blue lines* represent measurements just before bleach, *red lines* shortly after bleach, and *green lines* a few hours after bleach, when control animals are known to be fully dark-adapted. In the untreated rats, the pre (*blue*) and late-post (*green*) are similar in the Pmin region. The difference in the apparent thickness of the retina between these animals arises because the electrode does not always penetrate at the same angle to the retina. In the emixustat-treated animal, Pmin levels are elevated in the late-post bleach scan, indicating less oxygen consumption.

segments – approximately 40 μM from the choroid. The PO_2 rises across the outer nuclear layer as expected.[19] After the bleach in a control animal, the minimum PO_2, was higher as shown by the red profiles in Figure 4A. While the profile was recorded in darkness, this strong bleach kept channels closed and metabolism low until visual pigment regenerated, but recovery had been completed by 3.15 hours. Upon withdrawal of the electrode into the inner retina, the profiles were extremely variable, because the electrode sometimes was closer to blood vessels than at other times.

In the dark-adapted retina the profile had a trough in the outer retina, as shown, so it is easy to identify Pmin (Figs. 4A, 4B). However, in the light-adapted state, the profile sometimes had no trough, just a bend at the location of the inner segments, as in Figure 4B. This was not a drug effect, but was caused by variability in oxygen supply across the retina. It is known that the inner segments are at the same location in dark and light, so we continued to call the bend in the profile Pmin, because this was the location of Pmin in the dark. The retinal circulation influences Pmin, but in rat, the photoreceptors

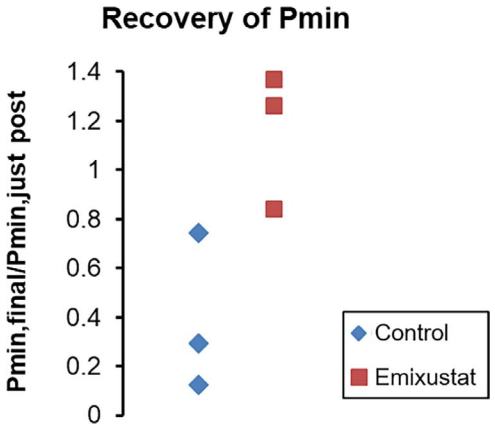

FIGURE 5. The ratio of Pmin in the final profile recorded to Pmin in the first profile after bleaching was calculated for each rat. The ratios varied, but values for untreated control animals (0.125, 0.293, and 0.745) were all lower than those for emixustat-treated animals (0.842, 1.263, and 1.370; $P = 0.035$), indicating higher oxygen levels and lower oxygen consumption in the emixustat-treated rats. For each group $n = 3$. Each point indicates an individual rat. A 2-tailed unpaired *t*-test was used to analyze statistical significance.

receive approximately 80% of their oxygen from the choroid,[19] so the influence of the choroid on Pmin is much stronger than the influence of the retinal circulation. From approximately 60 to 120 um, PO_2 was strongly dependent on where the profile is relative to the inner retinal blood vessels. Profiles were never repeated in exactly the same location twice; therefore, we focused on Pmin rather than the values throughout the profile.

In the animal treated with emixustat (Fig. 4B), Pmin did not return to the value that it had before bleach as it did in the control animal, but remained at the value just after bleach. Thus, it appeared that light adaptation was maintained while the retina was in the dark.

Minimum oxygen levels (PO_2 in mm Hg; Pmin) were assessed by measuring PO_2 before bleach, and at various times after bleach, within the retina at different distances from the choroid. Mean Pmin before bleaching was 8.6 mm Hg. It increased by 15.9 mm Hg at the first time point recorded after the bleach. The ratio of Pmin in the final profile recorded, divided by Pmin in the first profile after bleaching, was calculated for each rat (Fig. 5). The ratios varied, but values for untreated control animals (0.125, 0.293, and 0.745) were all lower than those for emixustat-treated animals (0.842, 1.263, and 1.370; $P = 0.035$), indicating greater recovery to the dark-adapted level in the control animals.

We normalized the Pmin values by dividing them by the choroidal PO_2 (Pc) values for each rat (Fig. 6). This ratio was considered a more stable measurement than Pmin alone, since rat photoreceptors receive approximately 80% of their oxygen from the choroid, thus, and Pmin is sensitive to Pc. For the control animals, the slopes of these lines were negative (–0.067, –0.088, and –0.108) and whereas the slopes of the lines for the emixustat-treated animals (0.015, 0.068, and –0.021) were virtually flat. This result was consistent with the hypothesis that emixustat treatment would delay dark adaptation and associated high oxygen use.

DISCUSSION

Emixustat depletes chromophore, which should generate apo-opsin. It was hypothesized that apo-opsin generated by emixustat treatment would initiate low level constitutive phototransduction. This would, in turn, reduce cGMP levels and ion channels would close, preventing cation influx, and reducing oxygen consumption. Our results supported this mechanism, showing that emixustat reduced cation influx during dark adaptation. These data indicated that emixustat can reduce the metabolically demanding dark current. We also demonstrated that PO_2 remains higher in dark-adapted emixu-

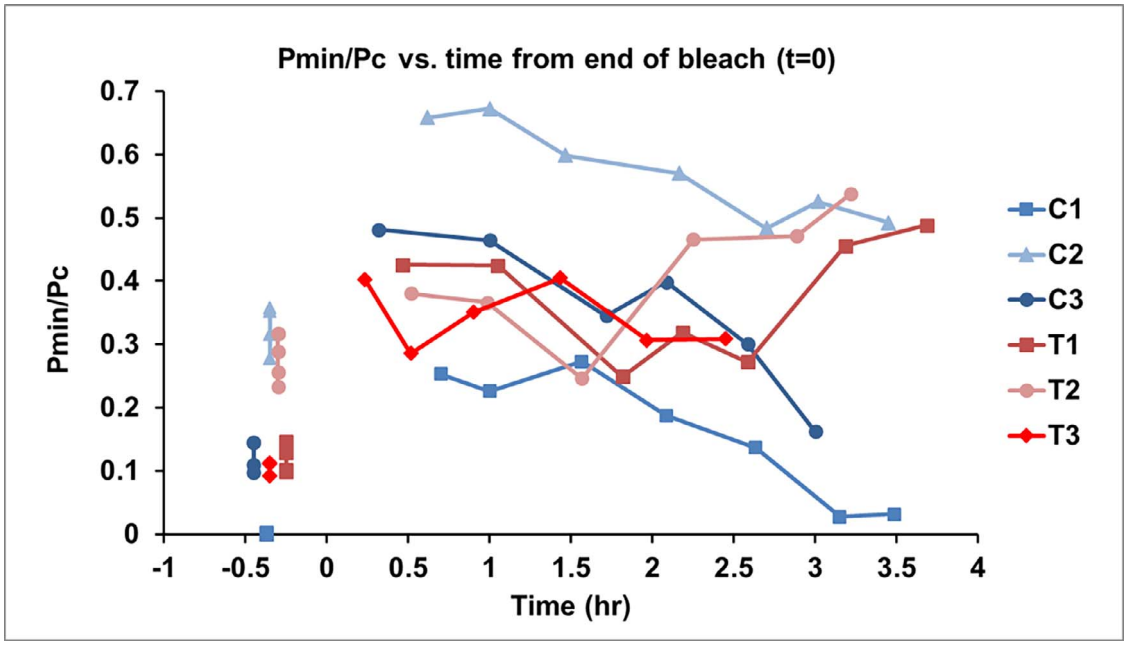

FIGURE 6. The Pmin values were normalized by dividing by the choroidal PO_2 (Pc) values. For the control animals, the slopes of these lines were negative, indicating decreasing oxygen level/increased oxygen consumption during dark adaptation. In the emixustat-treated animals the slopes were virtually flat line, indicating that oxygen levels were maintained, and less oxygen was consumed. Points at negative times show values of Pmin/Pc in several control dark-adapted profiles for each rat, and represent the expected value to which Pc/Pmin should eventually return in individual animals if oxygen consumption returns to its dark-adapted value. For each group, $n = 3$.

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stat-treated rat retinas compared to untreated dark-adapted retinas, indicating that that less oxygen was consumed in the photoreceptors of emixustat treated retinas. Together, these data showed that emixustat can reduce metabolic requirements of dark conditions.

Decreasing the metabolic demand of dark with emixustat may be therapeutic for diseases such as DR. However, because emixustat inhibits the visual cycle, there is a practical concern as to how emixustat treatment would affect vision. Emixustat seems to have no effect on vision from cone photoreceptors, which are responsible for high acuity central vision. Emixustat has been administered in 11 completed clinical studies for up to 2 years. Photoreceptor activity can be assessed by ERG. In completed clinical studies where ERG data are available, emixustat suppressed recovery of the rod b-wave amplitude following bleaching light exposure in a dose-dependent and reversible manner. Cone ERGs were not significantly affected.[8,20] In addition, visual acuity under normal, photopic conditions was not affected. The most common adverse events, visual color distortions and delayed dark adaptation, exhibited a dose-dependent trend in incidence and are consistent with emixustat's mechanism of action, with preferential effects on rod function. Effects on color perception likely occurred through secondary rod-cone interactions.

This finding that emixustat affects the rod ERG, but not the cone ERG ,may be attributed to the greater light sensitivity of rods, compared to cones. However, there is compelling evidence that cones do not rely on RPE65 as the primary source of visual chromophore and, therefore, may not be significantly affected by specific inhibitors of RPE65.[21,22] These observations led to the belief that visual pigment is regenerated at a faster rate in cones compared to rods and/or that chromophore may be supplied more efficiently to cones than to rods, possibly with the involvement of an alternate intraretinal visual cycle.

Recent progress in the characterization of the intraretinal visual cycle has shown that emixustat did not compromise the ability of cones to maintain light sensitivity during exposure to bright light.[23] Only the late, RPE visual cycle–driven phase of cone dark adaptation was suppressed by emixustat. From this and other findings, the investigators concluded that while visual pigment regeneration mediated by RPE65 appears to contribute to regeneration of cone pigments under certain conditions, RPE65 is not essential to maintain light sensitivity of cones.

Previously, we showed that emixustat reduced retinal neovascularization in the oxygen induced retinopathy model.[11] It was hypothesized that emixustat could reduce neovascularization by reducing metabolic stress. Other investigators hypothesized that prevention of complete dark adaptation, via activation of rod phototransduction, may be effective in preventing hypoxia and preserving retinal vasculature.[4,24] Data shown here support the hypothesized mechanism of action for the reduction of neovascularization. Decreasing retinal oxygen demand in the setting of diseases, such as DR, may reduce hypoxia associated with these conditions, potentially leading to a therapeutic effect. Emixustat may be promising potential treatment for ischemic retinal neovascularization such as DR.

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Disclosure: **R. Kubota**, Acucela (I, E, S), P; **D.J. Calkins**, Acucela (F); **S.H. Henry**, Acucela (I, E), P; **R.A. Linsenmeier** Acucela (F)

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